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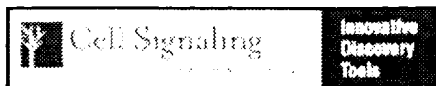
TITLE: Methods and compositions for inducing tumor-specific cytotoxicity

## DEPR:

Drug metabolizing enzymes which convert a pro-drug into a cytotoxic product include thymidine kinase (from herpes simplex or varicella zoster viruses), cytosine deaminase, nitroreductase, cytochrome p-450 2B1, thymidine phosphorylase, purine nucleoside phosphorylase, alkaline phosphatase, carboxypeptidases A and G2, linamarase, .beta.-lactamase and xanthine oxidase (see Rigg and Sikora, August 1997, Mol. Med. Today, pp. 359-366 for background).

## DEPR:

For example, a typical human dose of an adenoviral vector containing an H19 regulatory region operatively linked to a heterologous gene encoding a cytotoxic agent such as thymidine kinase is from 1.times.10.sup.7 pfu to 1.times.10.sup.10 pfu injected directly into the tumor mass per day. More preferably, the daily dose of such an adenoviral vector injected directly into a tumor would be from 1.times.10.sup.8 pfu to 1.times.10.sup.10 pfu, depending upon the tumor size. For an adenoviral vector containing an H19 regulatory region operatively linked to a cytotoxic gene product with a different level of toxicity, these values would of course be altered accordingly. Similar doses of an adenoviral vector containing an IGF-2 P4 promoter operatively linked to a heterologous gene encoding a cytotoxic agent such as thymidine kinase can also be used as a suggested starting point.

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## Dimerization, DNA Binding, and Transactivation Properties of Hypoxia-inducible Factor 1\*

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### ABSTRACT

Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric basic helix-loop-helix transcription factor that regulates hypoxia-inducible genes including the human erythropoietin (*EPO*) gene. In this study, we report structural features of the HIF-1 $\alpha$  subunit that are required for heterodimerization, DNA binding, and transactivation. The HIF-1 $\alpha$  and HIF-1 $\beta$  (ARNT; aryl hydrocarbon receptor nuclear translocator) subunits were coimmunoprecipitated from nuclear extracts, indicating that these proteins heterodimerize in the absence of DNA. *In vitro* translated HIF-1 $\alpha$  and HIF-1 $\beta$  were coimmunoprecipitated by

anti-HIF-1 $\alpha$  and anti-HIF-1 $\beta$  antibodies. Deletion analysis indicated that amino acids 1-590 mediated heterodimerization with HIF-1 $\beta$ . ARNT amino acids 1-590 were required for optimal DNA binding. A deletion involving the basic domain of HIF-1 $\alpha$  eliminated DNA binding without affecting

heterodimerization. In cotransfection assays, forced expression of recombinant HIF-1 $\alpha$  and HIF-1 $\beta$  (ARNT) activated transcription of reporter genes containing *EPO* enhancer sequences with intact, but not mutant, HIF-1 binding sites. Deletion of the carboxy terminus of HIF-1 $\alpha$  (amino acids 391-826) markedly decreased the ability of recombinant HIF-1 to activate transcription. Overexpression of a HIF-1 $\alpha$  construct with deletions of the basic domain and carboxy terminus blocked reporter gene activation by endogenous HIF-1 in hypoxic cells.

## INTRODUCTION

Multiple developmental and physiological mechanisms exist to provide each cell in the human body with sufficient O<sub>2</sub> to meet its metabolic demands. Essential to the maintenance of O<sub>2</sub> homeostasis is the production of adequate numbers of erythrocytes to transport O<sub>2</sub> from the lungs to peripheral tissues. Reduced O<sub>2</sub> delivery, whether due to anemia, acute hemorrhage, decreased ambient O<sub>2</sub> tension, or decreased O<sub>2</sub>-hemoglobin dissociation, is sensed by cells in the liver and kidney, which increase their synthesis of erythropoietin (EPO<sup>1</sup>), the glycoprotein hormone/growth factor that stimulates the survival, proliferation, and differentiation of bone marrow erythroid progenitor cells (reviewed in Refs. 1, 2, 3).

Analysis of *EPO* expression in the human hepatoblastoma line Hep3B has demonstrated that in cells subjected to hypoxia by incubation in 1% O<sub>2</sub>, *EPO* transcription is increased relative to nonhypoxic cells cultured in 20% O<sub>2</sub> (4, 5). DNA sequences in the human *EPO* gene 3'-flanking region functioned as a hypoxia-inducible enhancer in transient expression assays (reviewed in Ref. 6). A 50-bp 3'-flanking sequence mediated a 7-fold higher level of reporter gene expression in cells cultured at 1% compared to 20% O<sub>2</sub> (7). Mutational analysis indicated that the 50-bp enhancer was functionally tripartite (7).

Mutations at site 1 or site 2 eliminated enhancer function (7, 8). The first 33 bp of the enhancer (containing sites 1 and 2 only) functioned at one-half the level of the 50-bp element, but full activity could be restored by the presence of two copies of the 33-bp element, indicating that factors binding at site 3 amplified the induction signal but were not absolutely required for transcriptional activation (7). The orphan receptor hepatocyte nuclear factor 4 may bind at site 3 (9), the factor binding at site 2 is uncharacterized, and site 1 is bound by hypoxia-inducible factor 1 (HIF-1) (7).

Several lines of evidence indicate that HIF-1 plays a key role in *EPO* gene transcriptional activation in hypoxic cells. (a) A 3-bp substitution at site 1 eliminated enhancer activity and binding of HIF-1 (7). (b) Exposure of cells to 1% O<sub>2</sub>, cobalt chloride, or desferrioxamine induced both *EPO* expression and HIF-1 activity with similar kinetics (10, 11, 12). (c) Treatment of hypoxic cells with the protein kinase inhibitor 2-aminopurine or the protein synthesis inhibitor cycloheximide blocked induction of EPO RNA and HIF-1 activity (7, 10). In a variety of non-EPO-producing lines, including Chinese hamster ovary and human embryonic kidney 293 cells, HIF-1 was induced by hypoxia and *EPO* 3'-flanking sequences functioned as hypoxia-inducible enhancers (11, 13). Expression of genes encoding vascular endothelial growth factor and glycolytic enzymes was induced by exposure of EPO-producing and nonproducing cells to 1% O<sub>2</sub>, cobalt chloride, or desferrioxamine, and these genes contained HIF-1 binding sites within sequences mediating transcriptional activation (14, 15).

Purification of HIF-1 by DNA affinity chromatography (20) and characterization of amino acid and cDNA sequences revealed that HIF-1 was a heterodimeric transcription factor of the bHLH-PAS family

(21). The bHLH domain, present in a large number of transcription factors, mediates DNA binding and protein dimerization (reviewed in Refs. 22 and 23). The PAS (PER-ARNT-SIM) domain was described previously in the PER and SIM proteins of *Drosophila melanogaster* (24, 25) and the AHR and ARNT proteins, which constitute the mammalian dioxin receptor (26, 27, 28). PAS domains contain two internal homology units, the A and B repeats, and are implicated in protein-protein interactions (29, 30). The HIF-1 $\alpha$  subunit, consisting of 826 amino acids, was identified as a novel bHLH-PAS protein, whereas HIF-1 $\beta$  was identical to ARNT (21). ARNT, which is expressed as isoforms of 774 and 789 amino acids (27), can heterodimerize with HIF-1 $\alpha$ , AHR, or SIM and can also homodimerize (21, 31, 32, 33). HIF-1 $\alpha$  and HIF-1 $\beta$  mRNA and protein expression were induced in Hep3B cells exposed to 1% O<sub>2</sub> and rapidly decayed when cells were returned to 20% O<sub>2</sub>, consistent with the proposed role of HIF-1 in mediating transcriptional responses to hypoxia (21). Here we report a functional analysis of HIF-1 $\alpha$  and the identification of protein domains required for HIF-1 heterodimerization, DNA binding, and transcriptional activation.

## MATERIALS AND METHODS

### *Production of Glutathione S-Transferase (GST) Fusion Proteins*

Recombinant plasmids containing a HIF-1 $\alpha$  cDNA fragment (encoding amino acids 329-531) cloned into pGEX-3X and a HIF-1 $\beta$  (ARNT) cDNA fragment (encoding amino acids 496-789) cloned into pGEX-2T were constructed as described previously (21). Transformed *Escherichia coli* DH5 $\alpha$  cells were cultured in 50 ml of LB medium supplemented with 50  $\mu$ g/ml ampicillin at 37 °C at 200 rpm overnight, inoculated into 1 liter of LB medium supplemented with 50  $\mu$ g/ml ampicillin, and cultured at 37 °C at 200 rpm until  $A_{600} = 1.0$ . GST/HIF-1 $\alpha$  fusion protein synthesis was induced by adding isopropyl-1-thio- $\beta$ -D-galactopyranoside to 0.1 mM and shaking at 200 rpm at 30 °C for 1 h. The fusion protein was isolated as described (21), except that elution of protein was in 20 mM instead of 5 mM reduced glutathione. GST/HIF-1 $\beta$  fusion protein was induced and isolated as described (21). The affinity-purified fusion protein was analyzed by 10% SDS-PAGE and quantitated with a commercial kit (Bio-Rad).

### *Fusion Protein-Resin Preparation*

Purified fusion protein was coupled to hydrated cyanogen bromide-activated Sepharose 4B resin (Pharmacia Biotech Inc.) in 0.1 M NaHCO<sub>3</sub> (pH 9.0) and 0.5 M NaCl for 1 h at room temperature, according to the manufacturer's instructions. The coupling efficiency was greater than 95%, as determined by analyzing the unbound protein in the supernatant. Purified HIF-1 $\beta$  fusion protein (4.5 mg) was coupled to 2.5 ml of resin (0.71 g of freeze-dried powder), purified HIF-1 $\alpha$  fusion protein (0.82 mg) was coupled to 1 ml of resin (0.3 g of freeze-dried powder), and purified GST protein (2.5 mg) was coupled to 2.5 ml of resin (0.71 g of freeze-dried powder). After blocking with 0.1 M Tris-HCl, pH 8.0, and washing with 0.1 M sodium acetate, pH 4.0, 0.5 M NaCl, followed by 0.1 M Tris-HCl, pH 8.0, and 0.5 M NaCl, the gel was equilibrated and stored in Tris-buffered saline (TBS = 2.5 mM Tris-HCl, pH 8.0, 137 mM NaCl, and 2.7 mM KCl) supplemented with 0.5% (v/v) bovine serum albumin.

A 1:1:10 mixture (by volume) of anti-GST/HIF-1 $\beta$  antiserum/TBS/DH5 $\alpha$  cell lysate containing

GST/HIF-1 $\alpha$  (with a total protein concentration of 8.9  $\mu\text{g}/\mu\text{l}$ ) was incubated at room temperature for 1 h with agitation. A volume of GST-coupled Sepharose 4B equal to the volume of antiserum was added and incubated at 4 °C for 4 h. After centrifugation at 1500  $\times g$  for 3 min, the supernatant was combined with a volume of GST/HIF-1 $\beta$ -coupled Sepharose 4B equal to the volume of antiserum and incubated at 4 °C for 4 h with agitation. The resin was washed twice with 10 volumes of 50 mM sodium phosphate, pH 7.6, and 0.1% Triton X-100, then washed twice with 10 volumes of 10 mM sodium phosphate, pH 7.6. The adsorbed protein was eluted with 0.2 M glycine-HCl, pH 2.0, 0.1 M NaCl, and 0.1% Triton X-100 at room temperature for 15 min with agitation and collected in a tube containing 0.1 volume of 1 M Tris-HCl, pH 8.0, then dialyzed twice at 4 °C in 100 volumes of TBS for a total of 4 h.

#### *HIF-1 $\alpha$ Antibody Purification*

Anti-GST/HIF-1 $\alpha$  antiserum (1.2 ml) was incubated with 10.8 ml of TBS and 1.2 ml of GST-coupled Sepharose 4B for 4 h at 4 °C. After centrifugation at 1500  $\times g$  for 3 min, the supernatant was incubated with 1 ml of GST/HIF-1 $\alpha$ -coupled Sepharose 4B at 4 °C for 4 h. The resin was washed twice with 12 ml of 50 mM sodium phosphate, pH 7.6, and 0.1% Triton X-100; once with 12 ml of 10 mM sodium phosphate, pH 7.6, and 0.1% Triton; and once with 12 ml of 10 mM sodium phosphate, pH 7.6. The adsorbed protein was eluted and dialyzed as described above.

#### *Nuclear Extract Preparation and Immunoprecipitation*

Nuclear extracts were prepared from human Hep3B cells as described (7, 20). For immunoprecipitation, 400  $\mu\text{g}$  of nuclear extracts from cells exposed to 1 or 20% O<sub>2</sub> for 4 h were brought to a total volume of 1000  $\mu\text{l}$  with immunoprecipitation (IP) buffer (25 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 5 mM dithiothreitol, and 0.2% Nonidet P-40). Ten  $\mu\text{l}$  of preimmune serum were added and incubated for 2 h at 4 °C, followed by the addition of 200  $\mu\text{l}$  of a 50% suspension of protein A-Sepharose 4B (Pharmacia) in IP buffer for 1 h at 4 °C. The supernatant was collected by centrifugation at 5000  $\times g$  for 5 min at 4 °C and split into two tubes. Five  $\mu\text{l}$  of preimmune serum or 25  $\mu\text{l}$  of affinity-purified HIF-1 $\alpha$  polyclonal antibodies were added and incubated for 2 h at 4 °C, followed by incubation with 100  $\mu\text{l}$  of a 50% suspension of protein A-Sepharose 4B as described above. The IP of HIF-1 by HIF-1 $\beta$  antibodies was performed as above using 11  $\mu\text{l}$  of affinity-purified HIF-1 $\beta$  polyclonal antibodies. Pellets were collected by centrifugation for 5 min at 5000  $\times g$  at 4 °C, washed five times with 900  $\mu\text{l}$  of IP buffer, resuspended in 160  $\mu\text{l}$  of SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% (w/v) SDS, 2% 2-mercaptoethanol, and 10  $\mu\text{g}/\text{ml}$  bromophenol blue), heated at 98 °C for 5 min, and fractionated by electrophoresis through an SDS 7%-polyacrylamide gel. Immunoblot assay was performed as described (21), except that a 1:200 dilution of affinity-purified HIF-1 $\alpha$  antibodies or a 1:400 dilution of affinity-purified HIF-1 $\beta$  antibodies was used.

#### *Generation of Mutant Derivatives of HIF-1 $\alpha$*

Plasmid pBluescriptSK/HIF-1 $\alpha$ 3.2-3 (21) was digested with *Sma*I, *Hind*III, *Acl*I, *Afl*III, or *Pst*I and blunt-ended using Klenow fragment of DNA polymerase (Life Technologies, Inc.). Religation created a stop codon at the *Sma*I, *Hind*III, *Acl*I, *Afl*III, or *Pst*I site to generate the derivatives HIF-1 $\alpha$  $\Delta$ *Sma*I,  $\Delta$ *Hind*III,  $\Delta$ *Acl*I,  $\Delta$ *Afl*III, and  $\Delta$ *Pst*I, respectively. The basic domain of HIF-1 $\alpha$  was deleted by the replacement of an *Nco*I/*Bgl*II fragment in pBluescriptSK/HIF-1 $\alpha$ 3.2-3 with a double-stranded

oligonucleotide encoding the sequence of HIF-1 $\alpha$  C-terminal domain. This derivative was ligated into *Xba*I/*Xba*I-digested pCEP4 (Invitrogen) using T<sub>4</sub> DNA ligase (Stratagene). HIF-1 $\alpha$  $\Delta$ N $\Delta$ AB was

created by transfer of the HIF-1 $\alpha$ ΔNB sequences to pCEP4 and deletion of an *Afl*II-*Bam*HI fragment.

### *In Vitro Transcription and Translation*

The HIF-1 $\alpha$  cDNA and its mutant derivatives in pBluescriptSK contained either the T7 or T3 polymerase promoter in the appropriate orientation for *in vitro* expression. pBM5/Neo/M1-1 (27, 34), provided by Dr. Oliver Hankinson (University of California at Los Angeles), contained the T7 polymerase promoter for *in vitro* expression. *In vitro* transcription and translation was carried out using the TNT T7 or T3 coupled reticulocyte lysate system (Promega) in the presence or absence of L-[<sup>35</sup>S]methionine (Amersham Corp.) according to the manufacturer's instructions.

### *Electrophoretic Mobility Shift Assay (EMSA)*

EMSA using Hep3B nuclear extracts was performed with oligonucleotide probe W18 (7, 20). Unlabeled HIF-1 $\alpha$ , its mutant derivatives, and HIF-1 $\beta$  (ARNT) were synthesized *in vitro* as described above. EMSA using *in vitro*-translated proteins was performed as for nuclear extracts, except that binding reactions contained equal volumes of *in vitro* translation reactions and 2  $\times$  buffer Z+ (50 mM Tris-HCl (pH 7.5), 40% glycerol, 200 mM KCl, 0.4 mM EDTA, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1.2 mM sodium vanadate), 100 ng of calf thymus DNA, and 1  $\times$  buffer Z+ (25 mM Tris-HCl (pH 7.6), 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1.2 mM sodium vanadate) added to a final volume of 39  $\mu$ l. After preincubation for 5 min at room temperature, 1  $\mu$ l of labeled probe W18 (10<sup>4</sup> cpm) was added and incubated on ice for 15 min. Oligonucleotide competition experiments were performed with 1 ng or 10 ng of unlabeled W18 or M18 oligonucleotides. The sense strand sequences of the double-stranded W18 and M18 oligonucleotides are 5'-GCCCTACGTGCTGTCTCA-3' and 5'-GCCCTAAAAGCTGTCTCA-3', respectively. Supershift analysis was performed with the incubation of 1  $\mu$ l of preimmune serum or antiserum (21) against HIF-1 $\alpha$  at 1:3 dilution or HIF-1 $\beta$  at 1:6 dilution for 20 min on ice after probe addition.

### *Protein Dimerization Assay*

pBluescriptSK/HIF-1 $\alpha$ 3.2-3 and its mutant derivatives were transcribed and translated *in vitro* in the presence of [<sup>35</sup>S]methionine (Amersham). Aliquots (13  $\mu$ l) of labeled *in vitro* translation reactions of HIF-1 $\alpha$  or its derivatives were mixed with 13  $\mu$ l of unlabeled *in vitro* translation reactions programmed with pBM5/Neo/M1-1 and 26  $\mu$ l buffer Z+ and incubated at 30 °C for 15 min. At the end of the incubation, the reaction mixture was placed on ice for 30 min, incubated with 2  $\mu$ l of affinity-purified HIF-1 $\beta$  antibodies for 2 h at 4 °C, and then incubated with 13  $\mu$ l of 50% protein A-Sepharose 4B in IP buffer for 1 h. The pellets were washed five times with IP buffer and then heated for 5 min at 98 °C in SDS sample buffer. The immunoprecipitates and supernatants were analyzed by SDS 14% PAGE.

### *Transient Expression Assay*

Plasmid DNA was prepared using commercial kits (Qiagen). The construction of pSVcat reporter plasmids WT50, MUT50, and 2xWT33 has been described previously (7, 11). Cell culture of 293 cells and Hep3B cells was as described previously (7, 11). 293 cells were transfected by calcium phosphate precipitation (35). Hep3B cells were transfected by electroporation (36).

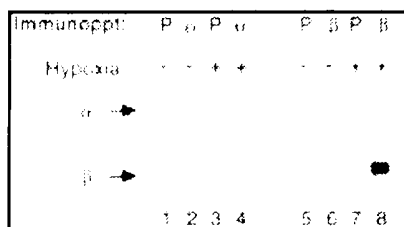
For dose-response studies, 293 cells were transfected with 1  $\mu$ g of pSVcat reporter plasmid (WT50, MUT50, or 2xWT33) and 0.1  $\mu$ g of pSV $\beta$ gal. For dose-response studies, 293 cells were transfected with 1  $\mu$ g of pSV $\beta$ gal and 0.8  $\mu$ g each of pCEP4 HIF-1 $\alpha$  and pBM5/Neo/M1-1 were mixed with 5  $\mu$ g of reporter

plasmid 2xWT33, and total plasmid DNA was adjusted to 22  $\mu$ g with pGEM4. Cells were cultured in 20 or 1%  $O_2$  for 36 h as described (7). HIF-1 $\alpha$  mutant derivatives were analyzed using 1  $\mu$ g of pSV $\beta$ gal, 5  $\mu$ g of 2xWT33, and 10  $\mu$ g of expression plasmid (5  $\mu$ g of pBM5/Neo/M1-1 and 5  $\mu$ g of pCEP4/HIF-1 $\alpha$ , pCEP4/HIF-1 $\alpha$  $\Delta$ PstI, or pCEP4/HIF-1 $\alpha$  $\Delta$ 47/11) or 10  $\mu$ g of pGEM4. Cells were cultured in 20 or 1%  $O_2$  for 40 h. Data were obtained from two independent experiments with three replications each. CAT and  $\beta$ gal values were determined as described (7). To test the dominant-negative form of HIF-1 $\alpha$ , Hep3B cells were transfected with 5  $\mu$ g of pSV $\beta$ gal, 10  $\mu$ g of 2xWT33-luciferase reporter and 0–40  $\mu$ g of pCEP4/HIF-1 $\alpha$  $\Delta$ NB $\Delta$ AB, and total expression plasmid DNA was adjusted to 40  $\mu$ g with pCEP4 DNA. Cells were incubated at 20%  $O_2$  for 24 h, followed by 24 h at 1%  $O_2$ . Luciferase activity was determined using 20  $\mu$ g of cell extract and 100  $\mu$ l of luciferase assay reagent (Promega).

## RESULTS

### Coimmunoprecipitation of HIF-1 $\alpha$ and HIF-1 $\beta$ from Crude Nuclear Extracts

We have demonstrated previously that antiserum raised against recombinant HIF-1 $\alpha$  or HIF-1 $\beta$  (ARNT) could supershift the HIF-1/DNA complex present in nuclear extracts of hypoxic Hep3B cells, suggesting that these proteins bind to DNA as a heterodimer (21). To demonstrate that HIF-1 $\alpha$  and HIF-1 $\beta$  associate in the absence of DNA, crude nuclear extracts from Hep3B cells were tested for coimmunoprecipitation of HIF-1 $\alpha$  and HIF-1 $\beta$ . Nuclear extracts were prepared from hypoxic and nonhypoxic Hep3B cells and incubated with affinity-purified antibodies against HIF-1 $\alpha$  or HIF-1 $\beta$  (ARNT). HIF-1-antibody complexes were precipitated with protein A-Sepharose 4B and fractionated by SDS-PAGE; HIF-1 $\alpha$  or HIF-1 $\beta$  protein was identified by immunoblot assay. As shown in Fig. 1 (*top panels*), HIF-1 $\alpha$  was immunoprecipitated from hypoxic nuclear extracts using antibodies raised against either HIF-1 $\alpha$  (Fig. 1, *lane 4*) or HIF-1 $\beta$  (Fig. 1, *lane 8*). HIF-1 $\alpha$  was detected as a series of isoforms as described previously (21) with an apparent molecular mass of approximately 113–131 kDa. HIF-1 $\alpha$  was undetectable by this assay when extracts from nonhypoxic cells were immunoprecipitated with HIF-1 $\alpha$  (Fig. 1, *lane 2*) or HIF-1 $\beta$  (Fig. 1, *lane 6*) antibodies, consistent with the extremely low levels of HIF-1 $\alpha$  detected in nonhypoxic Hep3B cells by direct immunoblot assay (21). No HIF-1 $\alpha$  was precipitated from hypoxic (Fig. 1, *lanes 3 and 7*) or nonhypoxic (Fig. 1, *lanes 1 and 5*) extracts using preimmune sera from the rabbits used for preparation of HIF-1 $\alpha$  and HIF-1 $\beta$  antibodies, demonstrating that the coimmunoprecipitation of HIF-1 $\alpha$  by HIF-1 $\beta$  antibodies was specific.



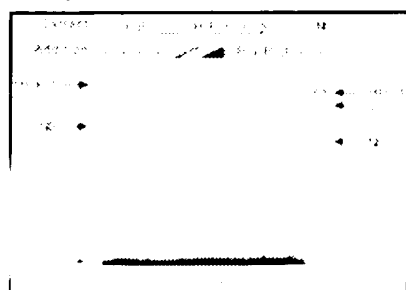
**Fig. 1. Coimmunoprecipitation of HIF-1 $\alpha$  and HIF-1 $\beta$  from nuclear extracts.** Aliquots of nuclear extracts from Hep3B cells exposed to 1%  $O_2$  for 4 h (+) or 20%  $O_2$  (–) were precleared by the addition of preimmune serum and precipitation with protein A-Sepharose 4B and incubated with affinity-purified HIF-1 $\alpha$  (*lanes 2 and 4*) or HIF-1 $\beta$  (*lanes 6 and 8*) antibodies, or the respective

preimmune serum (*lanes 1, 3, 5, and 7*), and precipitated by the addition of protein A-Sepharose 4B. Proteins in the precipitates from 100  $\mu$ g of nuclear extracts were fractionated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with a 1:200 dilution of purified HIF-1 $\alpha$  (*top panels*) or HIF-1 $\beta$  (*bottom panels*) antibodies.

As shown in Fig. 1 (*bottom panels*), HIF-1 $\beta$  was also immunoprecipitated from hypoxic nuclear extracts using either HIF-1 $\alpha$  (Fig. 1, *lane 4*) or HIF-1 $\beta$  (Fig. 1, *lane 8*) antibodies. HIF-1 $\beta$  was detected as isoforms with apparent molecular masses of approximately 100–107 kDa. After immunoprecipitation with HIF-1 $\alpha$  antibodies, HIF-1 $\beta$  was detected in hypoxic (Fig. 1, *lane 4*) but not in nonhypoxic (Fig. 1, *lane 2*) extracts. Preimmune serum precipitated small amounts of a protein from nonhypoxic (Fig. 1, *lane 1*) and hypoxic (Fig. 1, *lane 3*) extracts that was of similar mobility to HIF-1 $\beta$  and which cross-reacted with the anti-immunoglobulin conjugate used for detection of antibody-antigen complexes as it was visualized, even when the HIF-1 $\beta$  antibody was omitted from the immunoblot reaction (data not shown). HIF-1 $\beta$  was more easily detected when HIF-1 $\beta$  antibodies were used for immunoprecipitation (Fig. 1, *lane 8*) and a small amount of HIF-1 $\beta$  could be detected in immunoprecipitates from nonhypoxic extracts (Fig. 1, *lane 6*), consistent with previous direct immunoblot assays (21). HIF-1 $\beta$  was not immunoprecipitated by the preimmune serum from nonhypoxic (Fig. 1, *lane 5*) or hypoxic (Fig. 1, *lane 7*) nuclear extracts. Taken together, the results of these immunoprecipitation experiments indicate that HIF-1 $\alpha$  and HIF-1 $\beta$  are present as a heterodimer in nuclear extracts from hypoxic cells and suggest that not all HIF-1 $\beta$  exists as a heterodimer with HIF-1 $\alpha$ , especially in nonhypoxic nuclear extracts.

### Reconstitution of HIF-1 DNA Binding Activity by *in Vitro*-translated HIF-1 $\alpha$ and HIF-1 $\beta$

We next tested whether *in vitro* translation of HIF-1 $\alpha$  and HIF-1 $\beta$  proteins could reconstitute HIF-1 DNA binding activity. We performed EMSA using as probe a double-stranded oligonucleotide (W18) containing the HIF-1 binding site from the *EPO* enhancer (7). When unprogrammed reticulocyte lysates were assayed, a nonspecific DNA binding activity was detected by the probe (Fig. 2, *lane 1*). A similar pattern was seen when lysates were programmed with cDNA encoding HIF-1 $\alpha$  (Fig. 2, *lane 2*) or HIF-1 $\beta$  (Fig. 2, *lane 3*). However, when lysates were programmed with both HIF-1 $\alpha$  and HIF-1 $\beta$  (ARNT) cDNA (Fig. 2, *lane 4*), a new DNA binding activity was detected with mobility similar to that of HIF-1 present in nuclear extracts from hypoxic Hep3B cells (Fig. 2, *lane 14*). The recombinant HIF-1-DNA complex comigrated with the slower-migrating endogenous HIF-1-DNA complex. Glycerol gradient sedimentation analysis suggested that the faster- and slower-migrating complexes contain HIF-1 $\alpha$ /HIF-1 $\beta$  heterodimers and heterotetramers, respectively (20). As previously demonstrated for endogenous HIF-1 (7), binding of recombinant HIF-1 to the probe could be competed by increasing amounts of unlabeled W18 oligonucleotide (Fig. 2, *lanes 5* and 6) but not by unlabeled M18 oligonucleotide containing a 3-bp substitution within the HIF-1 binding site (Fig. 2, *lanes 7* and 8). The addition of antisera raised against HIF-1 $\alpha$  (Fig. 2, *lane 10*) or HIF-1 $\beta$  (Fig. 2, *lane 12*) to binding reactions containing *in vitro*-translated HIF-1 resulted in disruption of HIF-1-DNA complexes, whereas the respective preimmune serum had no effect (Fig. 2, *lanes 9* and 11). Thus, antisera specifically disrupted complexes containing recombinant HIF-1 rather than resulting in the supershift previously seen when crude nuclear extracts were analyzed (21). This difference may reflect the much lower protein concentrations in binding reactions containing *in vitro*-translated proteins rather than crude nuclear extracts. Taken together, the experimental results presented in Fig. 2 indicate that recombinant HIF-1 $\alpha$  and HIF-1 $\beta$  can reconstitute HIF-1 DNA binding activity *in vitro*, although the presence of a required cofactor in reticulocyte lysates cannot be excluded.



**Fig. 2. DNA binding specificity of *in vitro*-translated HIF-1 $\alpha$  and HIF-1 $\beta$  proteins.** Reticulocyte lysate (*lane 1*), HIF-1 $\alpha$  and HIF-1 $\beta$  translated individually (*lanes 2* and 3), or cotranslated (*lanes 4–13*) in

the presence of *in vitro*-translated HIF-1 $\alpha$  and HIF-1 $\beta$  and 50-fold excess competitor oligonucleotides and analyzed by EMSA. Antibody analysis was performed by preincubation of *in vitro* translation reaction aliquots

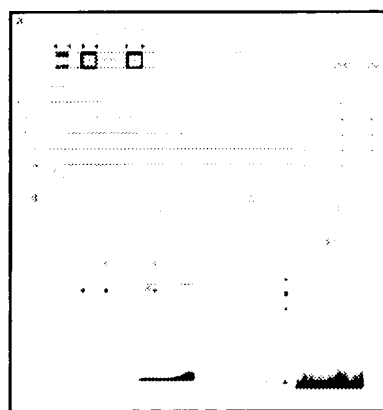


with W18 probe for 15 min on ice and incubation with no antiserum (*lane 13*), HIF-1 $\alpha$  preimmune serum (*lane 9*) and antiserum (*lane 10*), or HIF-1 $\beta$  preimmune serum (*lane 11*) and antiserum (*lane 12*). The mobility of endogenous HIF-1-DNA complexes was analyzed using 1  $\mu$ g of nuclear extract from hypoxic Hep3B cells (*lane 14*). Indicated at *left*: *rHIF-1*, recombinant HIF-1-DNA complex; *NS*, nonspecific DNA-binding activity; *FP*, free probe. Indicated at *right*: *HIF-1*, endogenous HIF-1/DNA complex; *C*, complex containing a constitutively expressed DNA binding activity (7).

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### Localization of HIF-1 $\alpha$ Dimerization and DNA Binding Domains

The demonstration that *in vitro*-translated HIF-1 $\alpha$  and HIF-1 $\beta$  could reconstitute HIF-1 DNA binding activity indicated that the recombinant proteins could heterodimerize *in vitro*. To demonstrate *in vitro* heterodimerization in the absence of DNA and to identify the HIF-1 $\alpha$  sequences required for dimerization and DNA binding, mutant derivatives were generated. HIF-1 $\alpha$  $\Delta$ StuI,  $\Delta$ HindIII,  $\Delta$ AccI,  $\Delta$ AflIII, and full-length (FL) HIF-1 $\alpha$  contained the amino-terminal 56, 166, 245, 390, and 826 amino acids of HIF-1 $\alpha$ , respectively (Fig. 3*A*). HIF-1 $\alpha$  $\Delta$ NB contained a deletion of amino acids 4-27, which overlap the basic domain that encompasses amino acids 17-30 (21). HIF-1 $\alpha$  and deletion mutants were translated *in vitro* in the presence of [ $^{35}$ S]methionine. The six proteins were synthesized in equal amounts, except for HIF-1 $\alpha$  $\Delta$ NB, which was synthesized with a higher relative efficiency (data not shown). The  $^{35}$ S-labeled proteins were each mixed with full-length unlabeled HIF-1 $\beta$  (ARNT). IP of labeled HIF-1 $\alpha$  by HIF-1 $\beta$  antibodies (pellet) indicated heterodimerization, whereas detection of labeled protein only in the supernatant indicated a lack of heterodimerization (Fig. 3*B*). HIF-1 $\alpha$  $\Delta$ NB dimerized with HIF-1 $\beta$  (Fig. 3*B*, *lane 4*), indicating that the basic domain is not required for heterodimerization. HIF-1 $\alpha$  $\Delta$ AflIII (Fig. 3*B*, *lane 5*),  $\Delta$ AccI (Fig. 3*B*, *lane 6*), and  $\Delta$ HindIII (Fig. 3*B*, *lane 7*) were also able to heterodimerize with HIF-1 $\beta$ , indicating that the HLH/PAS-A regions of HIF-1 $\alpha$  are sufficient for heterodimerization. HIF-1 $\alpha$  $\Delta$ StuI, which contained only a truncated bHLH region, did not dimerize with HIF-1 $\beta$  (Fig. 3*B*, *lane 8*).



**Fig. 3. Dimerization and DNA binding analysis of HIF-1 $\alpha$  deletion mutants.** *A*, structure of HIF-1 $\alpha$  constructs and summary of results.

*Box*, full-length HIF-1 $\alpha$  polypeptide with *arrows* indicating the first and last amino acid of bHLH domain and *A* and *B* internal homology units within the PAS domain (*hatched*); *thin line*, amino acid sequences of HIF-1 $\alpha$  constructs, with carboxyl-terminal amino acid residue indicated. Ability of each polypeptide to heterodimerize with full-length HIF-1 $\beta$  and bind to DNA is indicated (*right*). *B*, dimerization analysis of HIF-1 $\alpha$ . Each IP reaction contained an equal volume of unlabeled *in vitro*-translated HIF-1 $\beta$  (ARNT) (except in *lanes 1* and *2*) and

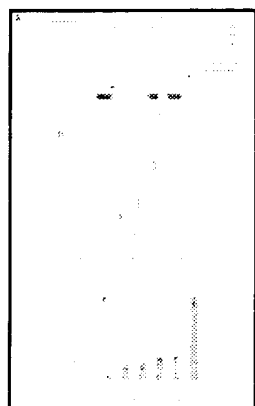
$^{35}$ S-labeled full-length HIF-1 $\alpha$  or the indicated mutant derivative (except in *lane 1*, which contains  $^{35}$ S-labeled *in vitro*-translated HIF-1 $\beta$  only). HIF-1 $\beta$  antibodies were used for IP. Pellet and supernatant were subjected to 15% SDS-PAGE and autoradiography. Migration of protein standards (mass in kDa) is indicated (*left*). *C*, DNA binding analysis of HIF-1 $\alpha$ . EMSA was performed using 6  $\mu$ l each of *in vitro*-translated HIF-1 $\beta$  and HIF-1 $\alpha$  or indicated mutant derivative, except in *lanes 1* and *2*, in which *in vitro*-translated HIF-1 $\beta$  only was used.

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After dimerization of unlabeled HIF-1 $\beta$  (ARNT) with unlabeled HIF-1 $\alpha$  or deletion mutant, the reactions were used to assay binding to the labeled W18 probe (Fig. 3C). As previously demonstrated, HIF-1 $\beta$  (Fig. 3C, lane 1) or HIF-1 $\alpha$  (Fig. 3C, lane 2) alone did not bind to W18, whereas DNA binding activity was detected in the presence of both full-length proteins (Fig. 3C, lane 3). In the presence of full-length HIF-1 $\beta$ , HIF-1 $\alpha$  $\Delta$ NB (Fig. 3C, lane 4),  $\Delta$ HindIII (Fig. 3C, lane 7) and  $\Delta$ StuI (Fig. 3C, lane 8) did not generate DNA binding activity, whereas a HIF-1-DNA complex was formed in the presence of  $\Delta$ AffII (Fig. 3C, lane 5) or  $\Delta$ AccI (Fig. 3C, lane 6), which generated protein-DNA complexes of increased mobility due to the truncation of HIF-1 $\alpha$ . These HIF-1/DNA complexes were competed by an excess of unlabeled oligonucleotide and were disrupted by HIF-1 $\beta$  antiserum (data not shown). These results indicate that DNA binding required the heterodimerization of HIF-1 $\beta$  with HIF-1 $\alpha$  containing an intact basic domain. The reduced and absent DNA binding associated with the  $\Delta$ AccI and  $\Delta$ HindIII mutants, respectively, suggest that an intact PAS domain is required for optimal binding of the HIF-1 $\alpha$ /HIF-1 $\beta$  heterodimer to DNA.

#### Transcriptional Activation by Recombinant HIF-1

To determine whether forced expression of HIF-1 $\alpha$  and HIF-1 $\beta$  (ARNT) could activate transcription, HIF-1 $\alpha$  and ARNT expression vectors were used to cotransfect cells with reporter plasmids containing *EPO* 3'-flanking sequences shown previously to function as a hypoxia-inducible enhancer (7, 11). SV40 promoter-CAT reporter plasmids (Fig. 4A) contained the wild-type 50-bp *EPO* enhancer (WT50) or a mutant enhancer (MUT50) containing a 3-bp substitution that was previously shown to eliminate enhancer function and that, when present in oligonucleotide M18, prevented HIF-1 binding (7). Reporter plasmids were cotransfected into Hep3B cells with an SV40 promoter- $\beta$ gal plasmid (pSV $\beta$ gal) in the presence or absence of HIF-1 $\alpha$  and HIF-1 $\beta$  (ARNT) expression vectors. Transfected cells were split onto two plates, incubated at 20% O<sub>2</sub> for 12 h, and then incubated at 1 or 20% O<sub>2</sub> for 24 h. CAT  $\beta$ gal ratios were normalized to those for the WT50 reporter in the absence of expression plasmids at 20% O<sub>2</sub>. WT50 expression was induced 13-fold by hypoxia in the absence of expression vector (Fig. 4B). In the presence of 0.5 and 5  $\mu$ g of expression vectors, there was a dose-dependent increase in CAT expression at both 20 and 1% O<sub>2</sub>. In contrast, MUT50 reporter gene expression was not induced significantly by hypoxia in the absence or presence of expression vectors. These results indicate that recombinant HIF-1 can activate transcription of reporter genes containing an *EPO* enhancer with an intact HIF-1 binding site.



**Fig. 4. Sequence-specific transactivation mediated by recombinant HIF-1.** A, structure of reporter plasmid constructs. WT50, 50-bp hypoxia-inducible enhancer from the *EPO* 3'-flanking region; MUT50, mutant enhancer sequence containing the indicated 3-bp substitution; WT33, first 33 bp of the 50-bp enhancer (7). One copy of WT50 or MUT50 or two copies of WT33 (2xWT33) were cloned 3' to the reporter plasmid transcriptional unit consisting of SV40 promoter, splice, and polyadenylation signals (stippled box) and CAT coding sequences (open box). B, transient cotransfection assay. Hep3B cells were cotransfected with 25  $\mu$ g of WT50 or MUT50 reporter plasmid, 3  $\mu$ g of pSV $\beta$ gal, and 0, 0.5, or 5  $\mu$ g of HIF-1 $\alpha$  and HIF-1 $\beta$  (ARNT) expression vectors. The cells were cultured in 20% O<sub>2</sub> for 12 h after transfection and then exposed to 20 or 1% O<sub>2</sub> for 24 h. CAT  $\beta$ gal

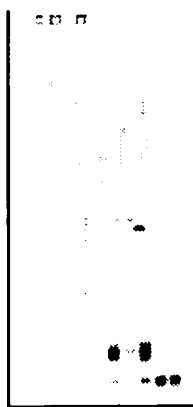
experiments, with each assay performed in duplicate. C, effect of increasing amounts of HIF-1 expression vectors on reporter gene expression. 293 cells were cotransfected with 5  $\mu$ g of 2xWT33 reporter

plasmid, 1  $\mu$ g of pSV $\beta$ gal, and 0, 0.5, 1, 2, 4, or 8  $\mu$ g of HIF-1 $\alpha$  and HIF-1 $\beta$  (ARNT) expression vectors. The cells were cultured at 20 or 1% O<sub>2</sub> for 36 h after transfection. Mean data were from two independent experiments with two replications each, and each assay was performed in duplicate; *bars*, S.E..  
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We also analyzed the effect of increasing amounts of HIF-1 $\alpha$  and HIF-1 $\beta$  (ARNT) expression vectors on transcription of the 2xWT33 reporter plasmid (containing two copies of the first 33 bp of the *EPO* enhancer) (Fig. 4A). For these experiments, we used 293 cells which, in contrast to Hep3B cells, do not express the *EPO* gene. In addition, we have shown previously that the response of reporter genes to hypoxia is more modest in 293 cells compared to Hep3B cells (11). Thus, these experiments provide analysis of a different reporter plasmid in a different cellular milieu. The CAT/ $\beta$ gal ratios were normalized to the result obtained in cells at 20% O<sub>2</sub> in the absence of expression vectors. There was a 2-fold increase in reporter gene expression in response to hypoxia in the absence of expression vectors (Fig. 4C). Relative CAT activity increased with the increasing amount of expression vectors used over the range of 0–8  $\mu$ g under both hypoxic and nonhypoxic conditions. The relative CAT activity was 7- and 21-fold higher in nonhypoxic and hypoxic cells transfected with 8  $\mu$ g of expression vectors, respectively, compared to cells transfected without the expression vectors. The large difference in CAT activity in 293 cells transfected with 4 and 8  $\mu$ g of expression vectors was not seen in Hep3B cells, where the effect of 4  $\mu$ g was intermediate between that of 2 and 8  $\mu$ g (data not shown). Taken together, the results in Fig. 4 indicate that recombinant HIF-1 can mediate sequence-specific and concentration-dependent transcriptional activation in both EPO-producing and -nonproducing cells.

#### *Analysis of Transcriptional Activation Mediated by HIF-1 $\alpha$ Deletion Mutants*

Expression vectors containing HIF-1 $\alpha$  mutant derivatives were created as illustrated in Fig. 5A. A translation stop codon was introduced at the *Pst*I and *Afl*III sites of HIF-1 $\alpha$  cDNA to generate HIF-1 $\alpha$  $\Delta$ *Pst*I and  $\Delta$ *Afl*III, respectively. HIF-1 $\alpha$  $\Delta$ NB $\Delta$ AB contained deletions of both the basic region (amino acids 4–27) and the carboxy terminus (amino acids 390–826) encoded by sequences distal to the *Afl*III site in HIF-1 $\alpha$  cDNA. Each mutant HIF-1 $\alpha$  expression vector was cotransfected into 293 cells with HIF-1 $\beta$  (ARNT) expression vector, 2xWT33 reporter, and pSV $\beta$ gal. CAT/ $\beta$ gal activity obtained for each HIF-1 $\alpha$  mutant construct was normalized to the values obtained in the absence of expression vectors at 20% O<sub>2</sub>. Expression of full-length HIF-1 $\alpha$  resulted in 7- and 29-fold higher levels of relative CAT activity at 20 and 1% O<sub>2</sub>, respectively, than in the absence of expression vectors (Fig. 5B). HIF-1 $\alpha$  $\Delta$ *Pst*I (amino acids 1–813), which lacked the last 13 amino acids at the carboxy terminus of HIF-1 $\alpha$ , activated significantly lower levels of CAT expression than full-length HIF-1 $\alpha$  with approximately 5- and 17-fold increases over control levels at 20 and 1% O<sub>2</sub>, respectively. HIF-1 $\alpha$  $\Delta$ *Afl*III (amino acids 1–390) mediated extremely reduced levels of reporter gene transactivation, with only 4- and 6-fold increases over control levels at 20 and 1% O<sub>2</sub>, respectively.



**Fig. 5. Functional analysis of HIF-1 $\alpha$  deletion mutants in cotransfection assays.** *A*, structure of mutant HIF-1 $\alpha$  proteins. *Box*, full-length (FL) HIF-1 $\alpha$ , amino acids 1-826;  $\Delta PstI$ , amino acids 1-813;  $\Delta A/I$ , amino acids 1-390;  $\Delta NB\Delta AB$ , amino acids 1-3 and 28-390. *B*, transient expression assay. 293 cells were cotransfected with 5  $\mu$ g of 2xWT33 reporter, 1  $\mu$ g of pSV $\beta$ gal, and 5  $\mu$ g of HIF-1 $\alpha$  or mutant derivative and HIF-1 $\beta$  expression vectors. The cells were cultured in 20 or 1% O<sub>2</sub> for 40 h after transfection. CAT/ $\beta$ gal activity was normalized to values obtained from cells in the absence of expression vector cultured at 20% O<sub>2</sub>. Mean data were derived from two independent experiments with three replications each; *bars*, S.E. ( $\chi^2$ ). *C*, DNA binding analysis of HIF-1 $\alpha$  mutants. 293 cells were transfected as in *B* but in the absence of reporter plasmid, incubated at 20% O<sub>2</sub> for 36 h, and then incubated at 20 or 1% O<sub>2</sub> for

4 h prior to nuclear extract preparation. EMSA was performed using 5  $\mu$ g of nuclear extracts and W18 probe. *D*, immunoblot assay. Fifteen- $\mu$ g aliquots of the same nuclear extracts analyzed in *C* were fractionated by SDS-PAGE, transferred to nitrocellulose membranes, and assayed for the presence of HIF-1 $\alpha$  (*top panel*) or HIF-1 $\beta$  (*bottom panel*) protein using affinity-purified antibodies.

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The reduced transactivation mediated by HIF-1 $\alpha\Delta PstI$  and  $\Delta A/I$  could be an indirect effect due to changes in protein expression, dimerization, or DNA binding activity. We, therefore, performed an EMSA using the W18 probe and nuclear extracts prepared from cells transfected with full-length HIF-1 $\beta$  (ARNT) expression vector and either vector only, full-length HIF-1 $\alpha$ , or one of the deletion mutants (Fig. 5C). Autoradiographic signals were quantitated by laser densitometry. In cells transfected with full-length HIF-1 $\alpha$  (Fig. 5C, *lanes 3 and 4*), there was 4-fold increased HIF-1 DNA binding activity at 20 and 1% O<sub>2</sub> compared to vector-transfected cells (Fig. 5C, *lanes 1 and 2*). Compared to control cells, HIF-1 DNA binding activity was 5-fold increased in cells transfected with HIF-1 $\alpha\Delta PstI$  (Fig. 5C, *lanes 5 and 6*). In cells transfected with HIF-1 $\alpha\Delta A/I$ , a new DNA binding activity with increased electrophoretic mobility was detected (Fig. 5C, *lanes 7 and 8*) which, as in the case of the *in vitro*-translated protein (Fig. 3C), represented probe complexes containing HIF-1 $\beta$  and the truncated HIF-1 $\alpha\Delta A/I$  protein. Remarkably, the DNA binding activity was much greater than that seen with any other construct, and equivalent levels of activity were seen in hypoxic and nonhypoxic cells, with 11-fold higher levels of DNA binding activity than in control hypoxic cells. These results indicate that the decreased transactivation mediated by HIF-1 $\alpha\Delta PstI$  and  $\Delta A/I$  was not due to reduced DNA binding activity and must, therefore, represent a specific loss of transactivation function.

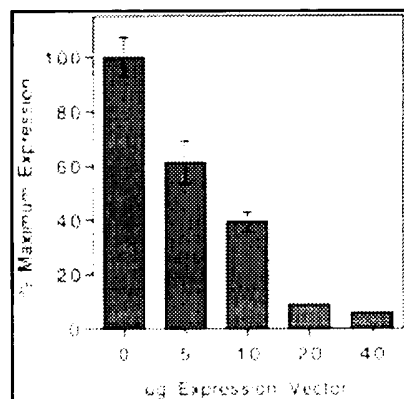
We also analyzed HIF-1 $\alpha$  and HIF-1 $\beta$  (ARNT) protein expression in the same nuclear extracts by immunoblot assay (Fig. 5D) and quantitation by laser densitometry. Transfection of full-length expression vectors resulted in increased levels of HIF-1 $\alpha$  protein at 20% O<sub>2</sub> (Fig. 5D, *top panel*, *lane 3*) and 1% O<sub>2</sub> (Fig. 5D, *top panel*, *lane 4*) compared to mock-transfected control cells (Fig. 5D, *top panel*, *lanes 1 and 2*). Increased levels of HIF-1 $\alpha$  protein were also detected in cells transfected with HIF-1 $\alpha\Delta PstI$  (Fig. 5D, *top panel*, *lanes 5 and 6*), and deletion of the last 13 amino acids did not appear to alter the electrophoretic mobility of HIF-1 $\alpha\Delta PstI$  relative to HIF-1 $\alpha$ FL. In hypoxic cells overexpressing HIF-1 $\alpha$ FL or HIF-1 $\alpha\Delta PstI$ , there were 20- and 29-fold higher levels of HIF-1 $\alpha$  than in control hypoxic cells. The HIF-1 $\alpha$  antibody recognizes both HIF-1 $\alpha$ FL and HIF-1 $\alpha\Delta PstI$ .

As expected, HIF-1 $\beta$  (ARNT) protein levels were also increased in cells transfected with full-length HIF-1 $\beta$  (ARNT)

and HIF-1 $\alpha$ FL (Fig. 5D, bottom panel, lanes 3 and 4) or HIF-1 $\alpha$  $\Delta$ PstI (Fig. 5D, bottom panel, lanes 5 and 6). As in the case of HIF-1 $\alpha$ , there was a greater increase in HIF-1 $\beta$  levels in transfected cells exposed to 1% O<sub>2</sub> (Fig. 5D, bottom panel, lanes 4 and 6) than in cells exposed to 20% O<sub>2</sub> (Fig. 5D, bottom panel, lanes 3 and 5), even when the induction of endogenous protein at 1% O<sub>2</sub> was taken into account. In cells transfected with HIF-1 $\beta$  (ARNT) and HIF-1 $\alpha$  $\Delta$ 47/II expression vectors, there was a dramatic increase in HIF-1 $\beta$  protein levels at both 20% O<sub>2</sub> (Fig. 5D, bottom panel, lane 7) and 1% O<sub>2</sub> (Fig. 5D, bottom panel, lane 8) to levels 21-fold higher than in hypoxic control cells. The analysis of HIF-1 $\alpha$  and HIF-1 $\beta$  protein expression thus paralleled the results obtained by EMSA (Fig. 5C'), indicating that the constitutively increased DNA binding activity in cells expressing HIF-1 $\alpha$  $\Delta$ 47/II and HIF-1 $\beta$  was due to constitutively increased levels of HIF-1 $\beta$  and, presumably, HIF-1 $\alpha$  $\Delta$ 47/II protein.

### Expression of a Dominant-negative Form of HIF-1 $\alpha$

We next investigated the effect of overexpressing HIF-1 $\alpha$  $\Delta$ NB $\Delta$ AB (Fig. 5A), which contains the basic domain deletion that affects DNA binding (Fig. 3C'), and the carboxyl-terminal truncation that affects transactivation (Fig. 5B). Based upon the results shown in Fig. 3, we hypothesized that this deletion mutant could heterodimerize with endogenous HIF-1 $\beta$ , generating biologically inactive heterodimers that would be unable to bind DNA and activate reporter gene transcription, thus competing with endogenous HIF-1 $\alpha$  for heterodimerization with HIF-1 $\beta$ . Hep3B cells were cotransfected with a constant amount of 2xWT33 reporter plasmid and pSV $\beta$ gal and increasing amounts of HIF-1 $\alpha$  $\Delta$ NB $\Delta$ AB expression vector along with the parental pCEP4 vector such that all cells received a total of 40  $\mu$ g of expression vector. In hypoxic cells, the activation of reporter gene expression by endogenous HIF-1 was inhibited by HIF-1 $\alpha$  $\Delta$ NB $\Delta$ AB in a concentration-dependent manner such that reporter gene expression in the presence of 40  $\mu$ g of HIF-1 $\alpha$  $\Delta$ NB $\Delta$ AB expression vector was reduced to 6% of the levels seen in cells transfected with 40  $\mu$ g of the parental pCEP4 vector (Fig. 6). These results provide further evidence that hypoxia-induced transcriptional activation of reporter genes containing the *EPO* enhancer is mediated by HIF-1.



**Fig. 6. Effect of a dominant-negative form of HIF-1 $\alpha$  on transcriptional activation in hypoxic cells.** Hep3B cells were cotransfected with 2xWT33-luciferase reporter, pSV $\beta$ gal, and increasing amounts of the expression vector pCEP4/HIF-1 $\alpha$  $\Delta$ NB $\Delta$ AB. Relative luciferase activity is normalized as a percentage of activity in cells transfected with pCEP4 vector only (column 1). Mean values are from three transfections; bars, S.E.

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## DISCUSSION

We have previously shown that the HIF-1 DNA complex generated by incubation of nuclear extracts from

hypoxic Hep3B cells with W18 probe contained proteins encoded by the cloned HIF-1 $\alpha$  and HIF-1 $\beta$  (ARNT) cDNA sequences (21). This finding did not rule out the possibility that HIF-1 $\alpha$ - and HIF-1 $\beta$ -bound DNA independently or heterodimerized only in the presence of DNA. We have now demonstrated by coimmunoprecipitation that HIF-1 $\alpha$  and HIF-1 $\beta$  (ARNT) exist as a heterodimer in the absence of DNA, as proposed previously based upon the results of glycerol gradient sedimentation analysis (20). We have also demonstrated that *in vitro*-translated HIF-1 $\alpha$  and HIF-1 $\beta$  (ARNT) can heterodimerize and reconstitute DNA binding activity with electrophoretic mobility, sequence specificity, and molecular composition similar to that of HIF-1 present in nuclear extracts of hypoxic Hep3B cells.

The results presented in this study allow localization of HIF-1 $\alpha$  sequences required for dimerization and DNA binding. As demonstrated previously for ARNT (34) and other bHLH proteins, we have shown that disruption of the HIF-1 $\alpha$  basic domain eliminates DNA binding without affecting heterodimerization. Previous analysis of ARNT deletion mutants also demonstrated that an intact HLH domain was necessary but not sufficient for dimerization with its alternative partner, AHR (34). A truncated ARNT protein consisting of the complete bHLH and PAS domains heterodimerized with AHR and recognized an AHR/ARNT binding site with high efficiency, whereas a truncated protein consisting of the bHLH and PAS-A domains showed reduced heterodimerization with AHR and greatly reduced DNA binding activity (34). Our analysis of HIF-1 $\alpha$  indicated that whereas amino acids 1-166, encompassing the bHLH and PAS-A domain, were sufficient for heterodimerization, optimal DNA binding of the HIF-1 $\alpha$ /HIF-1 $\beta$  heterodimer required the presence of HIF-1 $\alpha$  aa 1-390, encompassing complete bHLH and PAS domains. These results suggest that the presence of an intact PAS domain may be necessary to allow the basic domain of HIF-1 $\alpha$  (and perhaps HIF-1 $\beta$ ) to assume a proper conformation for DNA binding. It should be noted that the efficiency of heterodimerization and DNA binding by *in vitro*-translated HIF-1 $\alpha$  and HIF-1 $\beta$  was relatively modest, suggesting that posttranslational modification of one or both subunits, which occurs *in vivo* but not in reticulocyte lysates, is necessary for optimal heterodimerization and/or DNA binding. Alternatively, cofactor(s) present *in vivo* but not in reticulocyte lysates may be required for optimal activity.

### *HIF-1 Is a Transcriptional Activator*

Previous studies indicating that mutations which disrupted HIF-1 binding eliminated enhancer function (7) provided indirect evidence that HIF-1 was a transcriptional activator. In this study, we provide direct evidence from cotransfection assays that forced expression of HIF-1 $\alpha$  and HIF-1 $\beta$  (ARNT) is sufficient to activate transcription of reporter genes containing *EPO* enhancer elements with intact HIF-1 binding sites. In addition to binding site specificity, expression of reporter genes showed a dose-response relationship with respect to the amount of HIF-1 $\alpha$  and HIF-1 $\beta$  (ARNT) expression vectors that were cotransfected. Previous studies have identified transactivation domains at the carboxy terminus of AHR and ARNT (36, 37, 38). Cotransfection of full-length HIF-1 $\beta$  (ARNT) with truncation mutants of HIF-1 $\alpha$  suggest that a transactivation domain is located in the carboxyl-half of HIF-1 $\alpha$ . The deletion of amino acids 391-826 in HIF-1 $\alpha$  $\Delta 4/II$  decreased reporter gene activation to 13% of that observed with full-length HIF-1 $\alpha$  in cells at 1% O<sub>2</sub>, whereas at 20% O<sub>2</sub> HIF-1 $\alpha$  $\Delta 4/II$  retained 57% of the activity of full-length HIF-1 $\alpha$ . These results suggest that transactivation in cells at 1% O<sub>2</sub> is mediated primarily by the HIF-1 $\alpha$  carboxyl domain, whereas in cells at 20% O<sub>2</sub> another domain, such as the ARNT transactivation domain, plays an important role. As further evidence for a transactivation domain in HIF-1 $\alpha$ , we have recently demonstrated that a HIF-1 $\alpha$  carboxyl-terminal deletion mutant, HIF-1 $\alpha$  $\Delta 4/II$ , is

At all levels of expression vectors tested (including both wild-type and deletion mutants of HIF-1 $\alpha$ ),

reporter gene transcription was greater in cells at 1% than at 20% O<sub>2</sub>. Although these results may be explained in part by the greater expression of endogenous HIF-1 in cells at 1% O<sub>2</sub>, they also suggest that, in addition to the synthesis of HIF-1 $\alpha$  and HIF-1 $\beta$  protein, other hypoxia-induced events occur that are required for maximal transactivation by HIF-1. We have shown previously that HIF-1 $\alpha$  and HIF-1 $\beta$  mRNA and protein are extremely unstable in posthypoxic cells (21). Increased reporter gene expression in cotransfected cells cultured at 1% O<sub>2</sub> may, therefore, be due to stabilization of HIF-1 mRNA and/or protein in hypoxic cells. This conclusion is supported by the analysis of HIF-1 DNA binding activity and protein levels in transfected cells. In particular, the constitutively increased levels of DNA binding activity in transfected cells expressing HIF-1 $\alpha\Delta 47/11$  should be noted. This result implied high levels of HIF-1 $\alpha\Delta 47/11$  and HIF-1 $\beta$  protein in these cells. Although we could not determine HIF-1 $\alpha\Delta 47/11$  protein levels directly, there was a dramatic increase in HIF-1 $\beta$  levels that, in nonhypoxic cells transfected with HIF-1 $\alpha\Delta 47/11$ , were 22-fold higher than in cells transfected with HIF-1 $\alpha$ FL. Deletion of the carboxy terminus of HIF-1 $\alpha$  may increase its stability, similar to the effect of deleting the amino terminus of c-JUN (39). The carboxy terminus of HIF-1 $\alpha$  may target both HIF-1 $\alpha$  and HIF-1 $\beta$  for degradation, similar to the manner in which c-JUN targets both itself and its heterodimeric partner c-FOS for proteolysis (40). Pulse-chase experiments in cells expressing epitope-tagged proteins will be required to determine whether increased expression of HIF-1 $\alpha\Delta 47/11$  and HIF-1 $\beta$  protein is due to increased synthesis or decreased degradation.

We also demonstrated that a dominant-negative mutant, HIF-1 $\alpha\Delta$ NB $\Delta$ AB, which lacks both the basic DNA binding domain and carboxyl-terminal transactivation domain, could block transactivation of reporter genes containing the *EPO* enhancer in hypoxic cells, presumably by competing with endogenous HIF-1 $\alpha$  for heterodimerization with endogenous HIF-1 $\beta$ . Heterodimers of HIF-1 $\alpha\Delta$ NB $\Delta$ AB and HIF-1 $\beta$  are biologically inactive due to loss of DNA binding activity, as demonstrated *in vitro* for HIF-1 $\alpha\Delta$ NB. These results provide further evidence that the cloned HIF-1 subunits are involved in transactivation via the *EPO* enhancer and also provide an experimental paradigm through which it may be possible to analyze the biological effects of inactivating HIF-1 function *in vivo*.

## FOOTNOTES ■

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‡ The abbreviations used are: EPO, erythropoietin; bp, base pair(s); HIF-1, hypoxia-inducible factor 1; AHR, aryl hydrocarbon receptor; ARNT, AHR nuclear translocator; bHLH, basic helix-loop-helix; PAS, PER-ARNT-SIM; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; IP, immunoprecipitation; EMSA, electrophoretic mobility shift assay; CAT, chloramphenicol acetyltransferase;  $\beta$ -actin,  $\beta$ -actin;  $\Delta$ , deletion.

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